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Visualization of antigens attached to cytoskeletal framework in animal cells: Colocalization of simian virus 40 Vp1 polypeptide and actin in TC7 cells

(immunoelectron microscopy)

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ABSTRACT **Actin and the simian virus 40 viral structural polypeptide Vp1 are observed to be present on cytoskeletal fibers of virus-infected TC7 cells, when these antigens in detergent-extracted whole cell mounts were labeled by specific antibodies and colloidal gold particles coated with a second antibody. In both cases, actin and Vp1 were found associated with fibers and fiber-associated electron-dense materials. Patches or clusters of colloidal gold particles denoting the presence of either Vp1 or actin were found on fibers uniformly distributed throughout the cytoplasm. By using simultaneous decoration of the two antigens with colloidal gold particles of different diameters, it was shown that the majority of Vp1 appears attached to cytoskeletal fibers in association with cellular actin. When Vp1 and actin were decorated with Imposil and ferritin simultaneously in infected cells that were fixed first and then permeabilized with saponin, both labels were found in the same spatial domain of the cell cytoplasm. Thus, the colocalization of Vp1 and actin on the cytoskeletal framework seems to reflect their actual state in the living cells. The electron-dense material to which colloidal gold particles localize in our cytoskeletal preparations may be the remnants of subcellular structures with which actin and Vp1 are both associated in intact cells.**

The mechanism by which the transport of macromolecules from or to (or both) an interphase nucleus occurs is at present unknown. Experiments in which injected nuclear proteins redistribute rapidly from cytoplasm to nucleus, whereas injected bovine serum albumin stays in the cell cytoplasm, suggest that a specific mechanism is involved in determining the location of protein in cells (1–7). We have begun a study of the synthesis of proteins and their transport to the interphase cell nucleus using simian virus 40 (SV40) as a model system (8). During productive infection in permissive cells, progeny SV40 virions mature in the cell nucleus. The viral structural polypeptides are all synthesized in the cell cytoplasm and must be transported to the cell nucleus where virus assembly takes place. It has been shown that newly synthesized SV40 viral RNA is exclusively associated with the cytoskeletal framework (9) and that poly(A)$^+$ RNA isolated from a cytoskeletal fraction of SV40-infected cells directs the synthesis of SV40 structural polypeptides *in vitro* (10). These experiments suggest that the synthesis of SV40 polypeptides occurs on a skeletal framework.

The visualization by transmission electron microscopy of the detergent-insoluble fraction by using whole cell mounts has been reported (11–14). We have used cytoskeletal preparations to visualize viral as well as host cellular proteins by immunoelectron microscopy, and in this communication we describe the localization of SV40 Vp1 and actin antigens via antibodies bound to colloidal gold particles (CGP).

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MATERIALS AND METHODS

Cell lines, SV40 virus, the conditions for cell culture and virus infection, as well as the preparation of anti-Vp1 sera and specific anti-Vp1 IgG have been described (15). Monoclonal antibodies against actin (16) and against vimentin were generous gifts of J. Lin and L. B. Chen, respectively. Ferritin-coupled goat anti-mouse IgG and Imposil-coupled goat anti-rabbit IgG (17) were a generous gift of A. Dutton and A. H. Tokuyasu.

For the visualization of detergent-resistant cytoskeletons, whole cell mount procedures were used. Cells grown on formvar-coated gold grids supported by a glass coverslip were extracted with the lysis buffer (10 mM Tris·HCl, pH 7.2/250 mM sucrose/3.7 mM $CaCl_2$/12 mM $MgCl_2$/1% Nonidet P-40) at 4°C for 5 min. After two changes of this buffer they were further treated with the lysis buffer containing 0.15 M KCl for 3–5 min at 4°C, fixed either with 0.5% glutaraldehyde in phosphate-buffered saline [(P_i/NaCl) 137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8 mM Na_2HPO_4, pH 7.3] or with a combination of 0.2% glutaraldehyde and 1% water-soluble ethyldimethylaminopropyl carbodiimide as described by Willingham *et al.* (18), and treated with 5 mg of gelatin per ml in P_i/NaCl for 3 hr at 37°C. Cell preparations were reacted with mouse anti-actin antibody (at 1:500 or 1:6,000 dilution) or with 7.5 μg of rabbit anti-Vp1 IgG per ml for 45 min, rinsed with P_i/NaCl/0.1% Triton X-100 for 30 min, then rinsed with P_i/NaCl for 2 min, and reacted for 60 min either with goat anti-mouse IgG or with goat anti-rabbit IgG, which were coupled to CGP. Anti-actin or anti-Vp1 antibodies were diluted with P_i/NaCl/0.1% Nonidet P-40 containing 1 mg of normal goat IgG per ml and were centrifuged in a Microfuge for 2 min before use. Antibodies coupled to CGP were purchased from E. Y. Laboratory and were diluted with an equal volume of normal goat IgG (20 mg/ml in 2× concentrated P_i/NaCl) before the reaction. All immunoreaction steps and rinsing steps were performed at 37°C. The cell preparations were rinsed in P_i/NaCl/1% bovine serum albumin for 30 min, fixed with 1% glutaraldehyde in 0.1 M Na cacodylate (pH 7.2) at 4°C overnight, postfixed with 1% OsO_4 in 0.1 M Na cacodylate, dehydrated with serial concentrations of ethanol, dried from CO_2 by the critical point procedure, carbon coated, and examined in a Philips 301 transmission electron microscope operating at 80 keV.

Abbreviations: SV40, simian virus 40; CGP, colloidal gold particles; P_i/NaCl, phosphate-buffered saline.

RESULTS

Skeletal fibers remaining after the lysis of an epitheloid TC7 cell radiate from the nuclear area and extend to the periphery of the cell (Fig. 1). Other fibers crisscross the cytoplasm in a mesh-like manner. The bundles of fibers (mean ± SEM) around the nucleus and in the cytoplasm are 24.8 ± 0.7 nm (n = 39) and 21.1 ± 3.0 nm (n = 30) in diameter, respectively. The thickest fibers (Fig. 1) are composed mainly of intermediate filaments, because they were labeled by indirect immunofluorescence and immunogold procedures when cytoskeletal preparations were treated with monoclonal anti-vimentin antibody (see Fig. 3A). Often, regions containing webs of fine filaments (indicated by the arrow and the *Inset* in Fig. 1) are visible near the edge of well-spread cells. Such areas might be remnants of "ruffles" or possibly the sites of cell attachment to the substratum (19). Analyses of $NaDodSO_4$/polyacrylamide gel electrophoresis of proteins left after treatment with lysis buffer indicated that the prominent polypeptides remaining in detergent-insoluble fractions (cytoskeleton and nucleus) are those characteristic of intermediate filaments, actin, and histones. The major components of detergent-soluble fractions are actin and tubulin (data not shown). Similar but quantitatively different results were obtained by autoradiography of $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2) of the [^{35}S]methionine-labeled proteins recovered in the detergent-soluble, cytoskeletal, and nuclear fractions of TC7 cells before or after infection with SV40. The prominent radioactively labeled bands in soluble and nuclear fractions were those corresponding to actin and tubulin, and, in the cytoskeletal fraction, actin. An additional prominent band, Vp1, was observed in cytoskeletal and nuclear fractions of infected cells.

We were led to examine the localization of host and viral polypeptides directly on the cytoskeletal preparation by immunoelectron microscopy, because the radioactive Vp1 band was one of two prominent bands in the cytoskeletal fraction of infected cells (Fig. 2), reflecting its active synthesis. In addition, examination of the cytoskeletal preparation by immunofluorescence showed that Vp1 and actin produced overlapping staining patterns, which appeared as small patches throughout the cytoskeleton of the cell (Fig. 3 *C* and *D*).

Electron micrographs (Fig. 4) show cytoskeletal fibers decorated in part by CGP coupled to a second antibody, which binds either anti-actin (Fig. 4 *A* and *B*) or anti-Vp1 (Fig. 4 *C* and *D*). Actin is seen as patches associated with cytoskeletal fibers. The appearance of such patches is qualitatively the same in stained preparations that used different antibody concentrations (Fig. 4 *A* and *B*). Therefore, the patchiness was not due to inefficient labeling. Indirect immunofluorescence microscopy with monoclonal anti-actin also showed a staining pattern in the form of

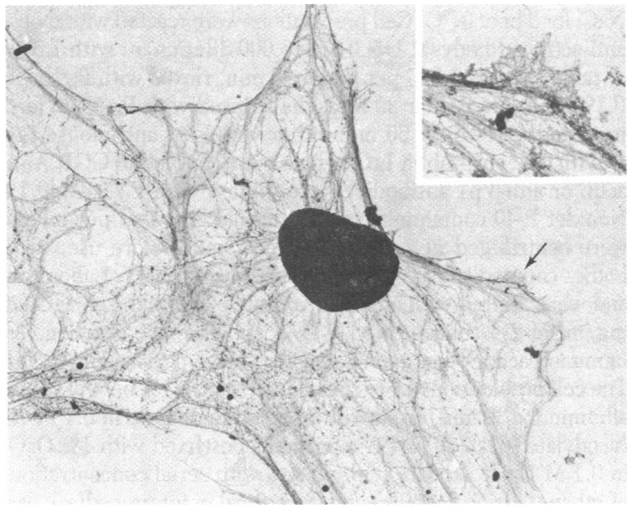

FIG. 1. Detergent-resistant cytoskeleton. The cytoskeleton was prepared by using whole cell mount procedures. The arrow indicates a fine mesh of filaments that are thinner than the majority of fibers. (×1,400.) (*Inset*) A higher magnification showing the web of fine filaments (arrow). (×4,900.)

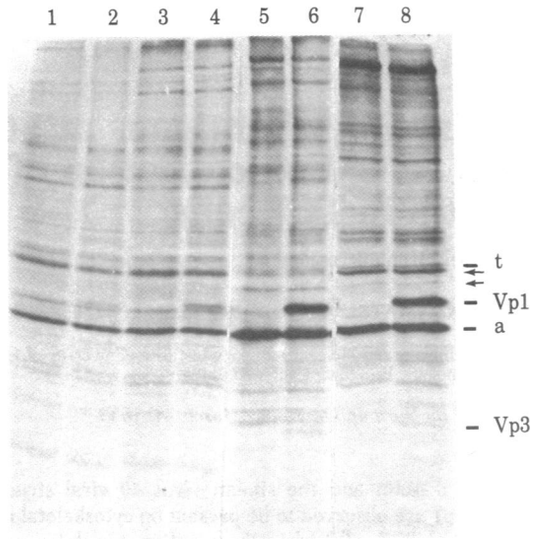

FIG. 2. $NaDodSO_4$/polyacrylamide gel of labeled subcellular fractions. Mock-infected or SV40-infected TC7 cells were grown in 60-mm culture dishes in medium containing 3% fetal calf serum, rinsed twice with TC7 media without methionine, and overlaid with 1 ml of the medium containing 0.25 mCi of [^{35}S]methionine (1 Ci = 3.7×10^{10} Bq) and 0.75 μg of methionine per ml. The dishes were agitated every 10 min. After 1 hr, 1 ml of TC7 media containing 3 μg of unlabeled methionine was added and the cells were incubated for an additional 2 hr. At the end of the labeling period, the cells were washed twice with TD buffer (0.14 M NaCl/5 mM KCl/0.7 mM Na_2HPO_4/2.5 mM Tris·HCl, pH 7.5) warmed to 37°C. All of the subsequent steps in fractionation were performed at 4°C. The cells were overlaid with 0.2 ml of the lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, and the soluble material was removed by pipet (soluble fraction). The residual cell material was overlaid with 0.15 ml of the lysis buffer containing 0.15 M KCl and was removed from the plate by scraping with a rubber policeman. The supernatant was separated by low-speed centrifugation (KCl-soluble fraction). The pellet containing the cytoskeletal network and nuclei was resuspended in 0.15 ml of buffer (10 mM Tris·HCl, pH 7.2/10 mM NaCl/1.5 mM $MgCl_2$/1% Tween 40/0.5% deoxycholate/1 mM phenylmethylsulfonyl fluoride) (RSB buffer/Tween/deoxycholate) (20), and homogenized in a Dounce homogenizer equipped with a B pestle for about 10–12 strokes. Homogenization separated most of the cytoskeletal network from the nuclei under these conditions. The integrity of the nuclei was monitored by light microscopy. The cytoskeletal and the nuclear fractions were then separated by low-speed centrifugation. The nuclei were resuspended in 40 μl of RSB buffer containing 150 μg of RNase A and 250 μg of DNase I per ml and were incubated for 15 min at 37°C. Six hundred thousand cpm of soluble (lanes 1 and 2), KCl-soluble (lanes 3 and 4), cytoskeletal (lanes 5 and 6), and nuclear fractions (lanes 7 and 8) of uninfected (lanes 1, 3, 5, and 7) or infected cells (lanes 2, 4, 6, and 8) were resuspended into Laemmli sample buffer and separated on a 12.5% $NaDodSO_4$/polyacrylamide gel. Band patterns of labeled polypeptides of the KCl-soluble fraction (lanes 3 and 4) were much more similar to those of soluble fractions (lanes 1 and 2) than those of cytoskeletal fractions (lanes 5 and 6). Bars and letters indicate where one would expect a polypeptide with a molecular weight identical to that for tubulin (t), Vp1 polypeptide (Vp1), actin (a), and Vp3 polypeptide (Vp3) to migrate. The arrows indicate bands comigrating with prominent intermediate filaments, which are insoluble in high salt (21). Note that an autoradiogram is illustrated, so that band density does not necessarily indicate abundance.

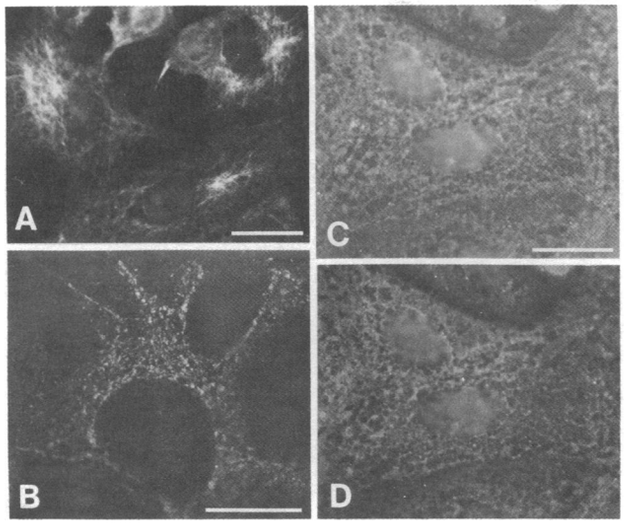

FIG. 3. Immunofluorescence micrographs of detergent-resistant cytoskeleton. Lysis buffer-extracted TC7 cells were stained with monoclonal anti-vimentin (*A*), monoclonal anti-actin (*B* and *D*), and rabbit anti-Vp1 (*C*). (*A* and *B*) Uninfected TC7 cells reacted with monoclonal antibodies and then reacted with rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA). (*C* and *D*) Double-immunofluorescence photograph of SV40-infected TC7 cells reacted with rabbit anti-Vp1 and mouse anti-actin and then reacted with fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories) and rhodamine-conjugated goat anti-mouse IgG. Vp1 staining (*C*) as well as actin staining (*D*) appear as patches. (Bars in *A* and *C* represent 50 μm; bar in *B* represents 25 μm.)

patches (Fig. 3*B*). In cells stained without prior extraction by lysis buffer, diffuse staining by anti-actin was seen by light microscopy. At the electron microscopy level Vp1, like actin, is found associated with fibers as clusters of gold particles (Fig. 4 *C* and *D*). In many cases, the CGP denoting presence of either actin or Vp1 are found associated with fibers and fiber-associated electron-dense materials. Patches or clusters of CGP are uniformly distributed throughout the cytoplasm, even in the areas including ruffles. The presence of the specific antibodies is essential for the decoration, because the omission of the specific antibodies results in seemingly nonspecific and much reduced labeling of the fibers (Fig. 4*E*).

Because both anti-Vp1 or anti-actin antibodies are found in fiber-associated electron-dense masses, we attempted the simultaneous detection of these antigens with CGP of different diameters. As shown in Fig. 5*A*, patches (arrows) intensely decorated with CGP were much more obvious than after actin (10-nm CGP) or Vp1 (14-nm CGP) decoration alone (Fig. 4 *B* and *C*). This is best seen at a higher magnification (Fig. 5*B*). To simplify discrimination between the two decorated antigens, we also used 38-nm CGP to decorate Vp1 along with 10-nm CGP to label actin. In 90% of the cases (n = 226 patches), Vp1 seen as a single or clustered 38-nm CGP was observed in the same patches as 10-nm CGP (denoting the presence of actin) (Fig. 5 *C–E*). Thus, the majority of SV40 structural polypeptide Vp1 detected is attached to cytoskeletal fibers in association with cellular actin in electron-dense patches. The efficiency of Vp1 decoration with antibody coupled to 38-nm CGP (Figs. 4*D* and 5 *C–E*) was much lower than that with antibody coupled to 14-nm CGP (Fig. 4*C*).

The cytoskeletal preparations used for this study were obtained after the extraction of soluble proteins by a nonionic detergent. Therefore, it is possible that the observed colocalization of Vp1 and actin does not reflect the actual state of these antigens in the living cells but occurred artifactually at the time of cell lysis. To determine if this was likely, uninfected or infected cells were first fixed with glutaraldehyde and water-soluble ethyldimethylaminopropyl carbodiimide to minimize intracellular rearrangement on lysis and only then were permeabilized with saponin. The preparations were treated with specific or control antibodies, followed by the second antibodies coupled either to ferritin or to Imposil (Fig. 5 *F* and *G*). Because of the thickness of these samples and the extensive retention of cytoplasmic components after these procedures, it was not feasible to examine whole cells. Therefore, thin sections were used instead.

Cells that were first reacted with mouse anti-actin and then with ferritin-conjugated goat anti-mouse IgG exhibited ferritin cores in the cytoplasm (Fig. 5*F*). The ferritin was often visible in association with electron-dense material in the cytoplasm. Similar results were obtained in uninfected cells (data not shown). The structures with which ferritin-decorated actin was associated could not be identified, because the morphology of the cytoplasm was highly altered by the permeabilization procedure. When Vp1 and actin were decorated with Imposil and ferritin simultaneously in infected cells, both labels were found in the same region of the cell (Fig. 5*G*). Control experiments showed that about 6% of the Imposil profiles had circular cross sections indistinguishable from ferritin. In experiments in which infected cells (n = 6) were labeled with both Imposil and ferritin via specific antibodies, 71% showed circular cross sections, thus indicating the simultaneous presence of Imposil and ferritin. Thus, these results again suggest that Vp1 and actin occupy the same spatial domain in the cell cytoplasm.

DISCUSSION

Using specific antibodies and a second antibody coupled to CGP, one can visualize proteins associated with cytoskeletal fibers of detergent-extracted whole cell mounts. This technique, when used with appropriate probes, should be useful not only for the anatomical delineation of proteins, but eventually also for other macromolecules, such as specific mRNAs on the cytoskeleton. It provides a mean for the study of the spatial arrangement of complex macromolecules in eukaryotic cells. We show here its use in localizing SV40 Vp1 and actin in the cytoskeleton of TC7 cells.

We have found that actin and Vp1 are found together in electron-dense structures associated with cytoplasmic filaments in the extracted cell cytoplasm. This could possibly reflect the attachment of nascent polypeptide chains and of the protein synthetic machinery to cytoskeletal fibers, as suggested by Lenk *et al.* (20) and by Fulton *et al.* (22). Our data do not allow us to distinguish between this hypothesis and one in which proteins synthesized elsewhere become anchored to the fibers. Because eventually some Vp1 becomes associated with the nucleus, it is tempting to view the association seen as a reflection of a transport function of cytoskeletal fibers. However, preliminary evidence (unpublished data) favors the idea that synthesis and transport are separate events.

The molecular composition of the cytoskeletal filaments observed in our preparation is not known with certainty, because we cannot associate a given protein seen on a gel with a particular fiber observed on the microscope screen. Our immunofluorescence as well as immunoelectron microscopic data suggest that they are intermediate filaments rather than microfilaments. Evidence not presented here and results obtained by other workers (23) suggest the presence of both vimentin and keratin in TC7 cells. In addition, fibers observed by electron microscopy were not well stained with the monoclonal actin antibody used in this study; yet this reagent has been shown to bind both to F and G actins (16). The same an-

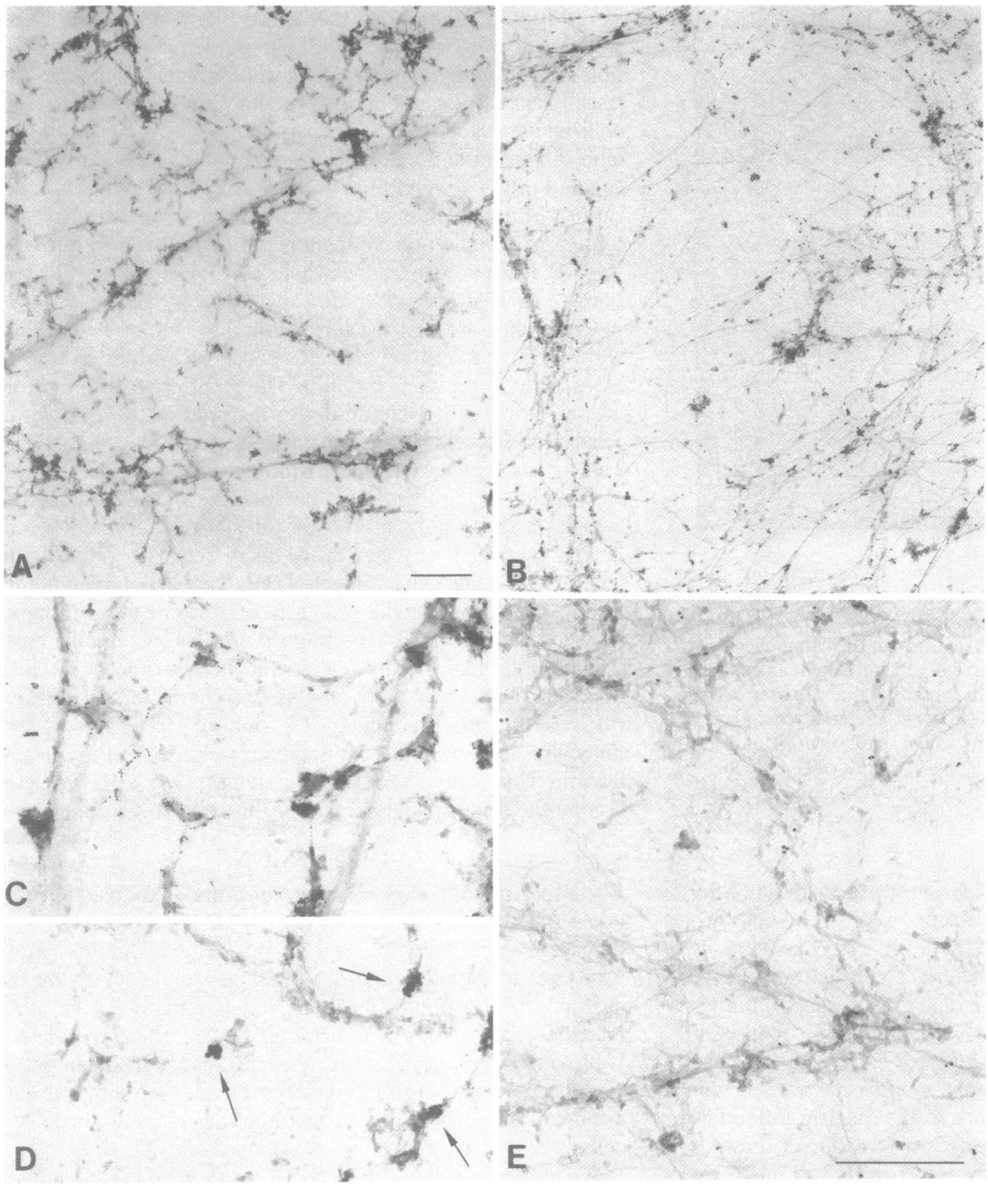

FIG. 4. Actin and Vp1 visualization on the cytoskeleton. Cytoskeletons of infected cells (46 hr after infection) were prepared and reacted with mouse anti-actin antibody at 1:6,000 dilution (*A*) or at 1:500 dilution (*B*), with rabbit anti-Vp1 antibody (*C* and *D*), or with normal goat IgG (*E*). Subsequent decorations of antigen–antibody complexes with CGP-coupled anti-antibodies were as follows: (*A* and *B*) goat anti-mouse IgG/10-nm CGP [10.5 ± 2.0 nm (mean ± SEM); n = 69]; (*C*) goat anti-rabbit IgG/14-nm CGP (14.5 ± 1.5 nm; n = 67); (*D*) goat anti-rabbit IgG/38-nm CGP (37.6 ± 8.8 nm; n = 24); and (*E*) goat anti-mouse IgG/10-nm CGP. The efficiency of Vp1 decoration with antibody coupled to 38-nm CGP (*E*) was much lower than that with antibody coupled to 14-nm CGP (*C*). The arrow in *D* points to a cluster of 38-nm CGP. *A–D* were photographed at the same magnifications. (Bars in *A* and *E* represent 0.5 μm.)

tibody reveals stress fibers in 3T3 cells by indirect immunofluorescence microscopy (J. Lin, personal communication; unpublished data), but in TC7 cells, only a diffuse cytoplasmic staining pattern could be seen (unpublished data).

Our immunoelectron microscopic observations differ in some respects from those of Webster *et al.* (14). These authors studied the detergent-resistant cytoskeleton of 3T3 and PtK2 cells. In their preparations, tubulin-containing fibers could be visualized and they could decorate some fibers with anti-actin. In our preparations, actin appeared associated with fibers and dense patches but did not seem to constitute the fiber itself. We interpret this difference as due to the buffer and the lysis conditions used to prepare the samples.

The colocalization of cellular actin with the viral polypeptide Vp1, both on cytoskeletal preparations and in cells that had been fixed and then permeabilized with saponin, is intriguing. The electron-dense material, with which actin and Vp1 were spatially associated in our cytoskeletal preparations, may be the remnants of subcellular structures to which actin and Vp1 both localize in intact cells. We cannot eliminate the unlikely alternative that two separate structures, one containing actin and the other containing Vp1, have collapsed, giving rise to the observed colocalization. The saponin permeabilization technique is too disruptive of cytological fine details to provide a definitive answer. The identification of the structure(s) to which the two proteins are attached will have to wait on improvement of the technique, when cytologically well-preserved material is thin-sectioned first and then immunolabeling procedures are carried out.

Preliminary experiments with anti-Vp3 and anti-actin also show these two antigens to colocalize (unpublished data). Pulse-labeled Vp1 and Vp3 also coisolate with pulse-labeled actin (unpublished data). Thus, there may be actin-containing structures that function in the synthesis or the transport, or both, of newly synthesized cellular proteins.

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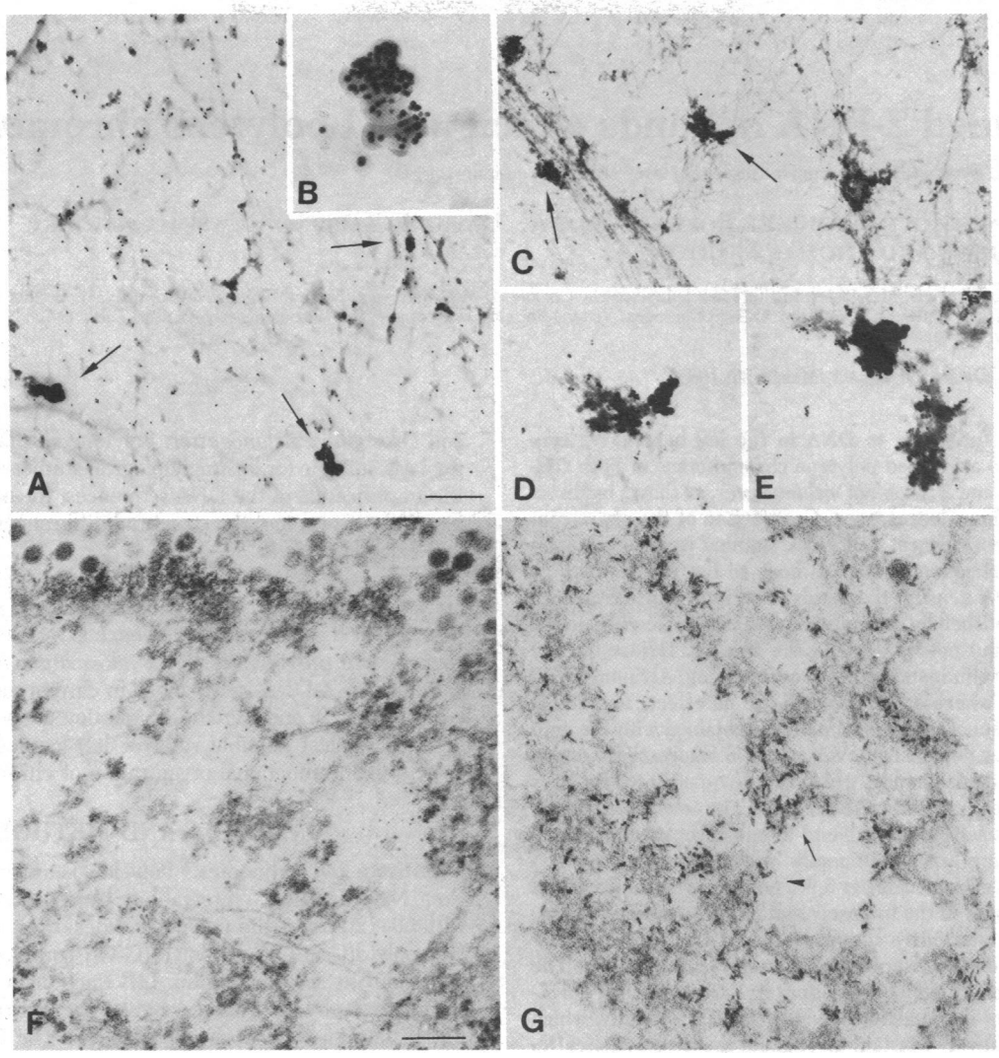

FIG. 5. Colocalization of actin and Vp1. (*A–E*) Detergent-resistant cytoskeletal preparations were reacted simultaneously with mouse anti-actin and rabbit anti-Vp1 and then were reacted with CGP-labeled second antibodies. Arrows in *A* and *C* point to clusters containing both CGP. (*D*) A higher magnification of a cluster shown in *C*. (*E*) A higher magnification of a cluster containing both 10-nm and 38-nm CGP. (*A* and *B*) 1:6,000 dilution of anti-actin/10-nm CGP goat anti-mouse IgG and anti-Vp1/14-nm CGP goat anti-rabbit IgG. (*C–E*) 1:500 dilution of anti-actin/10-nm CGP goat anti-mouse IgG and anti-Vp1/38-nm CGP goat anti-rabbit IgG. (*F* and *G*) Cells were grown on glass coverslips and infected with SV40 (10 plaque-forming units per cell). At 48 hr after infection cells were rinsed briefly, fixed with glutaraldehyde and water-soluble carbodiimide, permeabilized with saponin, and treated with normal goat IgG, as described by Willingham *et al.* (18). Cell preparations were reacted with mouse anti-actin antibody (1:1,000 dilution) (*F* and *G*) and with rabbit anti-Vp1 IgG (20 μg/ml) (*G*), rinsed for 90 min, reacted with ferritin-coupled goat anti-mouse IgG (*F* and *G*) or with Imposil-coupled goat anti-rabbit IgG (*G*), and rinsed extensively. All antibodies were diluted with saponin buffer (18) in 1 mg of normal goat IgG per ml. The stained preparations were postfixed in 1% glutaraldehyde, followed by 1% OsO_4, dehydrated with alcohol, and embedded in Epon. The thin sections (about 500 Å) were examined in a Philips 301 electron microscope at either 80 keV or 100 keV. The arrow and arrowhead in *G* indicate ferritin cores and Imposil, respectively. Photographs were taken at magnifications of 9,800 (*A* and *C*), 22,000 (*D* and *E*), and 43,000 (*B*, *F*, and *G*). Part of a nucleus containing virus particles is seen in *F*. Only cytoplasm shows in *G*. (Bars in *A* and *F* represent 0.50 μm and 0.25 μm, respectively.)

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